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13. ABSTRACT (Maximum 200 Words)
We hypothesize that simple interpretable patterns can be derived by following changes in breast cell from a single individual as they undergo progression from normal appearing ductular forms. We propose to: 1) Collect microdissected tissue representative of each of the morphologically different stages of early breast cancer progression in the MCF10AT model to obtain RNA and DNA for miroarray analysis of gene expression and PCR-amplification for analysis of global and gene specific CpG island methylation, 2) Compare methylation patterns of candidate genes in tissue with those of the cultured MCF10AT derived lines used for xenografts, 3) Prepare a CpG island methylation and a gene expression profile for each of the tissue types and cell lines.
This year, we completed examination of DNA methylation and mRNA levels of 7 candidate genes by bisulfite sequencing and quantitive methylation specific PCR in 6 lines including MCF10CA lines with differing metastatic potential. Methylation status of 3 genes was determined in microdessected DCIS formed by these lines. No major differences were detected between cell lines but significant changes in methylation occurred in tumors. Microarray analysis of DNA methylation and expression to be carried out in the next year should aid interpretation of these differences.

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I. INTRODUCTION:

There is a growing literature that suggests a causal role for aberrant DNA methylation in breast cancer development and a number of breast cancer cell lines have been shown to carry genes silenced by methylation. Genes that are up- or down- regulated in human breast cancers are already being studied by microarray gene expression analysis, and several studies of changes in methylation at CpG islands in cultured breast cells and breast tumor samples have been reported. However, even though classification of tumors on the basis of gene expression patterns and methylation profiles are being derived from candidate genes in a variety of tumors, the genetic, epigenetic, cellular and morphological heterogeneity of the breast tumor samples derived from different patients greatly complicates derivation of mechanistic explanations of tumor development from patient material. Thus, it would be of great utility to be able to follow the changes in breast tissue from single individuals as breast cancer develops and progresses.

Our objective in these studies is to develop a profile of epigenetic and gene expression changes occurring during the early stages of breast cancer progression. Our hypothesis is that a simpler, interpretable pattern of changes, similar to that developed for progression of colon cancer in individuals with familial polyposis coli can be derived by studying the changes that occur in breast cells from a single individual as they undergo change from normal appearing ductular forms, through hyperplasia to the appearance of carcinoma-in-situ and locally invasive cancer. The aims are to: 1) Collect enough microdissected tissue representative of each of the morphologically different stages of early breast cancer progression in the MCF10AT model to allow preparation of RNA for microarray analysis of gene expression and PCR-amplification of DNA for analysis of global and gene specific CpG island methylation. 2) To compare the methylation patterns of candidate genes in these tissues with those of MCF10A, MCF10AT and MCF10DCIS.com, cells grown in culture. 3) To prepare a methylated CpG island profile for and a gene expression profile for each of the tissue types and cell lines.

II. BODY:

Task 1. Optimize techniques for analysis of gene array and CpG island analysis using cultured MCF10AT cells. (Months 1-12) Completed

a. Determine minimum number of cells needed to accurately detect differential expression of candidate genes by microarray.
b. Determine minimum number of cells required to accurately detect differences in CpG islands by array .
c. Sequence CpG islands that demonstrate differential cross-hybridization with RT-PCR products from cultured MCF10A and MCF10AT and MCF10DCIS.com cells.

Task 1 a and b were completed in months 1-12. In task b, we determined that ~ 2 μg of DNA (~2 x 10^6 cells) was required to provide sufficient probe for one array. This is well within the number of cells we can obtain from our tissue culture experiments. However, it was simple to calculate that this much material could not be reasonably obtained by laser capture, even with

access to the most efficient laser capture microscope currently available (Leica). Such equipment allows isolation of >150,000 cells in less than a day when the sample is large enough.

To address this problem, we have developed a novel technique during the last year for producing probe sufficient for multiple arrays by first amplifying ~50-100 ng of DNA (20 rounds), cleaving with restriction endonucleases to allow addition of a new linker with a T7 polymerase binding site at one end and using this material as template for producing an average of 10-12 μg of fluorescence-tagged RNA probe. We have just initiated our analysis of CpG island arrays using these novel probes to detect CpG islands whose methylation is altered in the cultured cells and xenografts. We are in the in the process of comparing results obtained with DNA and RNA probes using material from cultured MCF10AT family cells. (See Task 4 below.)

Our studies last year indicated that we could easily carry out quantitative methylation specific PCR (Methylight) [1] with as little as 24 pg of DNA isolated from microdissected xenografts. We have now finished evaluation of DCIS lesions formed in xenografts from three MCF10 lines (See Task 2 below).

Our strategy in the coming year will be to complete identification of potentially important differentially methylated CpG islands (Task 1 c) on our 40,000 CpG island clone arrays using PCR products from cultured cells and xenografts. Methylation specific PCR of bisulfite modified DNA will be used to confirm the methylation of novel and known CpG islands in morphologically different regions of microdissected MCF10AT tumors.

Task 2. Collect sufficient xenograft tissue to begin analysis of progression in xenografts (Months 2-15).

a. Quantitate and optimize recovery of cells from defined areas of lesions.
b. Quantitate recovery of DNA and RNA from microdissected tissues
c. Optimize RT-PCR and CpG island PCR from microdissected tissue.
d. Inject 50 N/B mice in each flank with MCF10AT cells and another 50 with MCF10DCIS.com cells. Collect lesions at times indicated in methods

Task 2. a and b were completed in months 1-12 and, as noted above, we have optimized both RT-PCR and CpG island PCR from microdissected tissues. We have collected the areas with the morphology of carcinoma *in situ* (DCIS) in tumors formed by MCF10DCIS.com as well as several other highly malignant breast tumor lines derived from MCF10AT cells by Dr. Fred Miller at Karmanos Inst.[2, 3] We have analyzed methylation of the APC, CDH1 and GSTP1 in all 6 cell lines and 2 genes in the microdissected tumor tissues from MCF DCIS.com, MCF10CA1, MCF10CA1d and MCFC10Alh) using methylation specific PCR.

In our reference DNA, isolated from MCF7 cells, the APC CpG island is sparingly methylated and the GSTP1 CpG island fully methylated at the sites examined. In all of the collected tissue identified morphologically as carcinoma *in situ*, regardless of the cell line utilized, the APC CpG island remained unmethylated. However, the GSTP1 CpG island was partially methylated in DCIS from all four lines. Since GSTP1 is unmethylated in the cells used to form the tumors, at a minimum, this result suggests that some CpG islands can become

preferentially methylated during xenograft tumor formation or that there is selection of a subpopulation of cells during tumor development. These are questions that can be addressed in future work.

REAL TIME PCR C_T VALUES FOR METHYLIGHT DETECTION OF DNA METHYLATION IN 8 pg OF DNA ISOLATED FROM CULTURED BREAST CELL LINES.

Probe	*β-actin*	*APC Methyl.*	*APC Unmethyl.*	*GSTP1 Methyl.*	*GSTP1 Unmethyl*	*CDH1 Methyl.*	*CDH1 Unmethyl*
DNA Source							
mDNA	39.9	36.7	0	38	0	37.9	0
unDNA	37.9	0	37.5	0	39.8	0	39.7
MCF10Ca1a	36.6	0	37.3	0	38.1	0	37.3
MCF10Ca1d	38.6	0	38.3	0	40.3	0	38.3
MCF10Ca1h	38.4	0	38.8	0	39.2	0	39.1
MCFDCIS.com	37.8	0	37.8	0	38.9	0	37.3
MCF7	36	38.1	37.1	36.6	0	0	38.6

Note: The numbers shown are the average of three determinations of the CT value for detection of fluorescence released from the indicated probe (The smaller the value the higher the extent of methylation. mDNA=normal lymphocyte DNA methylated with SssI. UnDNA=untreated normal lymphocyte DNA. β-actin is used to normalize the sample DNA quantity using primers/probes that are not sensitive to changes in methylation.

REAL TIME PCR C_T VALUES FOR METHLYLIGHT DETECTION OF DNA METHYLATION IN MICRODISSECTED TUMOR TISSUE.

Probe	*β-actin*	*APC Methylated*	*APC Unmethyl.*	*GSTP1 Methylated*	*GSTP1 Unmethyl.*
DNA Source					
mDNA	37.4	35.5	0	41.5	0
unDNA	37.4	0	37.8	0	40.1
MCF10CA1a	33.8	0	34.9	41.3	38.5
MCF10CA1d	33.5	0	33.6	42.7	38.7
MCF10CA1h	34.2	0	32.6	43.5	38.7
MCFDCIS.com	35.8	0	35.8	39.5	40.2
MCF7	37.8	36.3	37.4	36.4	0

Task 3. Complete analysis of methylation and expression of candidate genes in cultured MCF10A, MCF10AT and MCF10DCIS.com.

As noted last year, we made excellent progress on this aim. In months 13-24 it is near completion. We focused on determining the extent of methylation and expression of a set of genes known to be hyper-methylated in cancer, using cultured cells of the MCF10 model system for breast cancer progression. Since we intend to carry out extensive studies on both cultured cells and tumors using CpG island microarray, we picked a limited set of genes to allow comparison of the results obtained by CpG island microarray with those obtained by methylation specific PCR and those obtained by the "gold standard" bisulfite sequencing. This is important to do because CpG island microarray, by nature, will only detect loss or gain of methylation at sites recognized by restriction endonucleases whose cleavage is blocked by methylation at CpG sites (BstUI, HpaII, etc). Methylation specific PCR allows a rapid check on methylation status for known CpG islands but is more limited than the CpG island microarray in terms of the size of the area that can be examined. The results of this part of the study have been important not only from the aspect of obtaining new information about methylation of these genes in cell lines representative of different stages in breast cancer progression but also for interpreting data obtained by CpG island microarray [4].

1. *CpG Island microarray analysis:* We have made "mini-microarrays" of CpG islands of 16 tumor suppressor genes known to be hypermethylated in cancer (APC, p14 ARF, E-cadherin, p16, p15, estrogen receptor, glutathione s-transferase P1, HIC1, MGMT, MLH1, RB, RIZ1, BRCA1, TMS1, TIMP3 and NOEY2 as well as controls (cloned mitochondrial and ribosomal DNA and MYOD1). In each case, the cloned fragment was from a region flanked by MseI sites. This is the same restriction enzyme site used for cloning CpG islands during preparation of the CpG island library [5]. As reported last year, our results indicated that BRCA1 and E-cadherin CpG islands were fully methylated even in the non-tumorigenic MCF10A cells, giving an equivalent signal with PCR probe synthesized either before or after cleavage with BstUI. This signal was similarly to that of mitochondrial DNA (no BstUI sites) and ribosomal RNA (highly methylated). CDH1, GSTP1, NOEY2, p16, BRCA1, HIC1, RBL,TIMP3 and RIZ1 appeared to be methylated, while ESR1and p15 were unmethylated. This array pattern did not differ between the MCF10 derived lines, i.e, during the establishment of the lesion-forming MCF10AT line from MCF10A or selection of more malignant derivatives of MCF10AT. However, although methylation of BRCA1 and E-cadherin "makes sense" in terms of breast tumor biology, the cloned regions from the CpG islands in both genes contained Alu repeats which have the potential to anneal with the PCR products amplified from Alu repeats elsewhere in the genome. This would give a strong "methylated" signal, since the Alu repeats are generally highly methylated. When the Alu repeats were removed from the CpG island sequences spotted on the microarray both genes appeared to be unmethylated (data not shown). This was confirmed using methylation specific PCR in a region close to the transcription start site. We therefore decided to do a complete bisulfite sequencing analysis of the promoter/CpG island regions of a small number of relevant genes.

2. *Analysis of DNA methylation of candidate genes by methylation specific PCR and sequencing of bisulfite treated DNA.* As reported last year, we completed examination of CpG island methylation of four genes using bisulfite sequencing of DNA from MCF10A and the six tumor cell lines derived from it. Methylation of TIMP3 (tissue inhibitor of metalloproteins); WT1

(Wilm's tumor suppressor gene); NOEY2 (a member of the ras superfamily) and APC (Adenomatous Polyposis Coli) was confirmed using both methylation specific PCR and bisulfite sequencing. We now report completion of the bisulfite sequencing of GSTP1, CDH1 and BRCA1 from all the MCF10A lines. We have also sequenced a second upstream CpG island of NOEY2. The same bisulfite sequencing information was obtained from MCF7 cells to allow comparison with data published by others. The results are summarized in Table 3 below.

3. *Expression of APC, TIMP3, NOEY2 and WT1 mRNA in MCF10A and MCF10A derived lines*
In the report submitted for mos. 1-12, we presented the results of analysis of mRNA levels by standard RT-PCR amplification (35 cycles) followed by gel electrophoresis and Southern blotting. This semi-quantitative analysis of RT-PCR products (PCR of various mRNAs compared to GAPDH as reference gene) suggested that TIMP3 and APC are expressed at relatively high levels in all cell lines and in normal breast tissue, that WT1 is expressed at a barely detectable level in all of the cell lines (but not in the normal breast tissue) and that NOEY2 is highly expressed in two of the MCF10CA lines and normal breast but is expressed at relatively low levels in MCF10A,10AT and two different MCF10CA lines.

To confirm and extend these observations, quantitative real time RT Q-PCR was carried out using standard protocols [6]. The data was analyzed using GAPDH as the reference gene and MCF10A, the non-tumorigenic precursor of the other MCF10 lines as the "calibrator sample" for determining the relative level of expression of each individual gene. The data (below) are consistent with but differ somewhat from the standard RT-PCR evaluation. These data are included in the summary Table 3. This summary shows that:
The APC gene CpG island, which lies between bases –216 to –20 of the transcription start site is unmethylated in all MCF10 derived lines and MCF7. All lines have low mRNA expression levels for APC ($2^{-\Delta\Delta CT}$ <1). Conclusion: Methylation at these 20 sites is not strongly implicated in silenced expression.
The NOEY-2 gene has two CpG islands. Our earlier study focused on the region from –315 to +217). This island was heavily methylated in MCF10A but not in its derived lines. The mRNA expression level was very low in all lines ($2^{-\Delta\Delta CT}$ <0.2) suggesting that methylation in this region is not required for silencing. However, the upstream CpG island (-1120 to –864) is completely methylated in all MCF10 cell lines, suggesting that methylation in this region may lead to silencing. This island is represented in our microarray and NOEY-2 is detected as methylated in all of the MCF10 lines by microarray.
The WT1 gene CpG island is methylated at all 56 sites in all cell lines, including MCF7 and mRNA is expressed at low levels (($2^{-\Delta\Delta CT}$ <0.2) in all lines. MCF7 expresses mRNA at somewhat higher level with a $2^{-\Delta\Delta CT}$ of 0.875. Again, this suggests a role of methylation in WT1 silencing.
The TIMP-3 gene CpG island (-105-+312) is heavily methylated at the majority of its 66 CpG sites in MCF10A lines but is still expressed at high levels ($2^{-\Delta\Delta CT}$ ≥47) in the metastic MCF10Ca1h line. Thus, methylation in this region does not silence expression.
The GSTP1 gene CpG island (-336 to +106) is unmethylated and expressed in all of the MCF10A lines and methylated and unexpressed in MCF7 as expected.
The BRCA1 gene CpG island is variably methylated in MCF10A lines in the regions (–647 to +500) downstream of the Alu repeats (See Chart 1 for BRCA1 CpG island methylation). The BRCA1 Alu repeat region is highly methylated in all lines (-1435 to -1099). None of the lines

express BRCA1 at high levels ($2^{-\Delta\Delta CT}$ >4), whether methylated or not in the region between –1052 and +86, suggesting that either transcription is suppressed by methylation of the Alu repeat or that regulation does not involve methylation. We are searching for a line known to express normal levels of BRAC1 to clarify this point.

The CDH1 gene CpG island is completely unmethylated in all MCF10A lines and in MCF7 but mRNA is expressed at relatively low levels($2^{-\Delta\Delta CT}$ ≤4.4) in MCF10A lines compared to MCF7 ($2^{-\Delta\Delta CT}$ >40). This CpG island also has an Alu repeat region upstream. We are currently examining methylation of this area to see if it differs between the expressing MCF7 and the non-expressing MCF10A lines.

It should be emphasized that at least five of CpG islands we have studied in detail have been implicated by other groups as being sites for methylation mediated silencing of these genes in breast cell lines or tumors [7-12] . We have either used the same primers/probes or new primers in the same region that were developed to improve the efficiency of PCR and our PCR results (MS-PCR) are generally consistent with bisulfite sequencing (Summary Table I.) Thus, while we can clearly concluded that there is little or/no change in the methylation of the genes regions we have studied during the derivation of the MCF10A model system for progression, there are still significant differences in the expression levels of the mRNAs encoded by the genes adjacent to these CpG islands. We also know that changes in methylation occur during tumor formation in xenografts. Examination of gene expression in the microdissected tissues is underway and will be completed in the coming year.

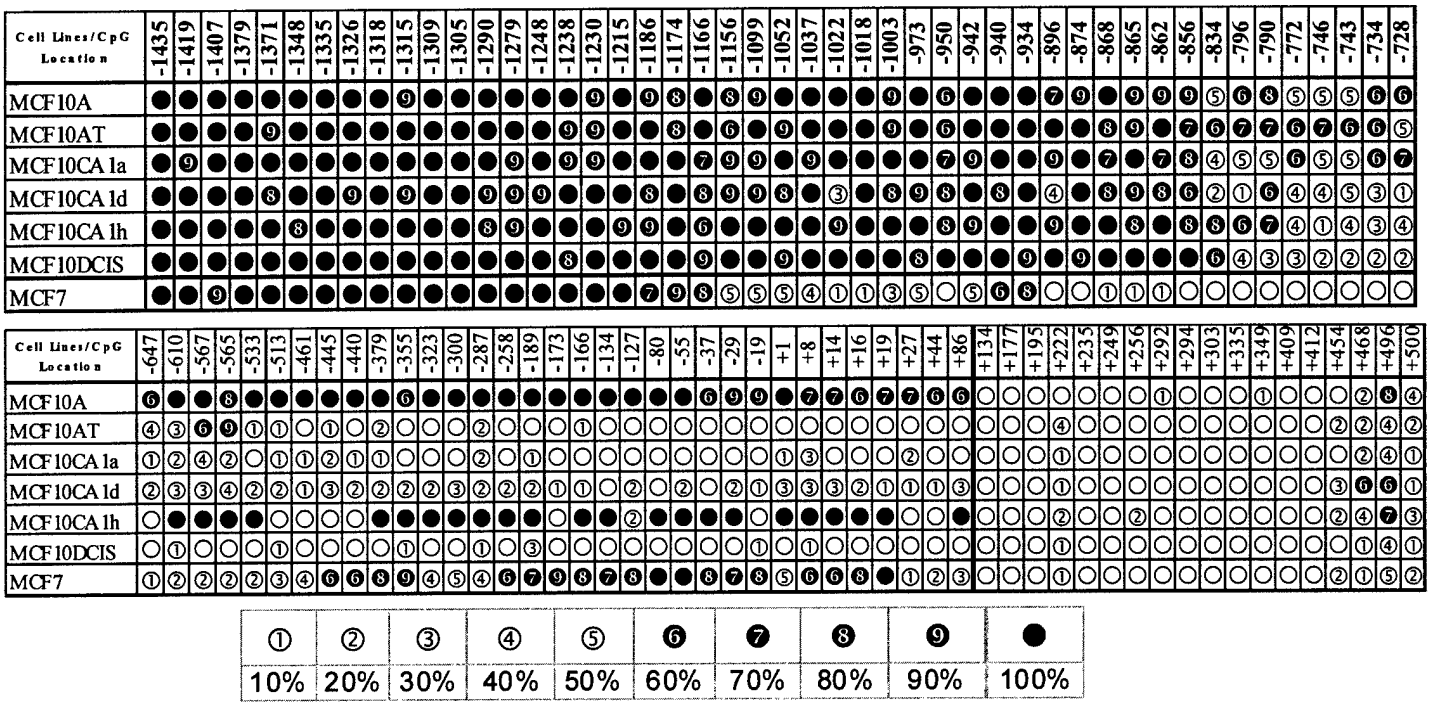
Summary Table I

GENE	MS-PCR	Bisulfite Sequencing (CpG sites)*		Expression level+ $2^{-\Delta\Delta CT}$
APC		-216 to –20		
MCF-10A	U	U(<10%M at 1/20 random sites)		0.33
MCF-10AT	U	U		0.245
MCF-10CA1a	U	U		0.182
MCF-10CA1d	U	U		0.131
MCF-10CA1h	U	U		0110
MCF-10DCIS	U	U		0.117
MCF7	U/M	M(10-20% M random at 19/20 sites)		0.55
NOEY-2		I (-1120 to-864)	II (-315 to +217)	
MCF-10A	M	M	M (≥50%M at 27/30 sites)	0.002
MCF-10AT	M	M	U (≤30% M at 28/30 sites)	0.004
MCF-10CA1a	M	M	U	0.0235
MCF-10CA1d	M	M	U	0.016
MCF-10CA1h	M	M	U	0.050
MCF-10DCIS	M	M	U	0.115
MCF7	M	M	ND	0.002

WT1		(-105 to +312)	
MCF-10A	M	M (≥90%M at 56 sites)	0.002
MCF-10AT	M	M	0.004
MCF-10CA1a	M	M	0.0235
MCF-10CA1d	M	M	0.016
MCF-10CA1h	M	M	0.050
MCF-10DCIS	M	M	0.115
MCF7	M	M	0.002
TIMP-3		(-224 to +313)	
MCF-10A	M	M (59/66 sites>80% M)	4.1
MCF-10AT	M	M	5.3
MCF-10CA1a	M	M	0.6
MCF-10CA1d	M	M	11.3
MCF-10CA1h	M	M	46.7
MCF-10DCIS	M	M	24.8
MCF7	U	U (4/66 sites <20% M)	3.9
CDH-1		(-363 to +86)	
MCF-10A	U	U (≤30% at all 34 sites)	4.4
MCF-10AT	U	U	5
MCF-10CA1a	U	U	4.2
MCF-10CA1d	U	U	3.8
MCF-10CA1h	U	U	0.8
MCF-10DCIS	U	U	2.4
MCF7	U	U	41
BRCA1		(See Chart I below)	
MCF-10A	U	M/U	2.4
MCF-10AT	U	M/U	2.0
MCF-10CA1a	U	M/PM	2.1
MCF-10CA1d	U	M/U	2.0
MCF-10CA1h	U	M/PM	0.7
MCF-10DCIS	U	M/U	0.8
MCF7	U	M/PM	2.64
GSTP1		(-336 to +106)	
MCF-10A		U (≤1%M at all 51 sites)	24.13
MCF-10AT		U	24.64
MCF-10CA1a		U	7.33
MCF-10CA1d		U	7.37
MCF-10CA1h		U	5.08
MCF-10DCIS		U	4.83
MCF7		M (>80% at 49/51 sites)	0

*(bases flanking the CpG island examined are indicated as are the number of CpG sites in the region.) +Relative gene expression for the total set relative to MCF10A determined as described in Ref. [13]. M, methylated; PM, partially methylated; U unmethylated.

Chart I. Bisulfite sequencing of BRCA1.



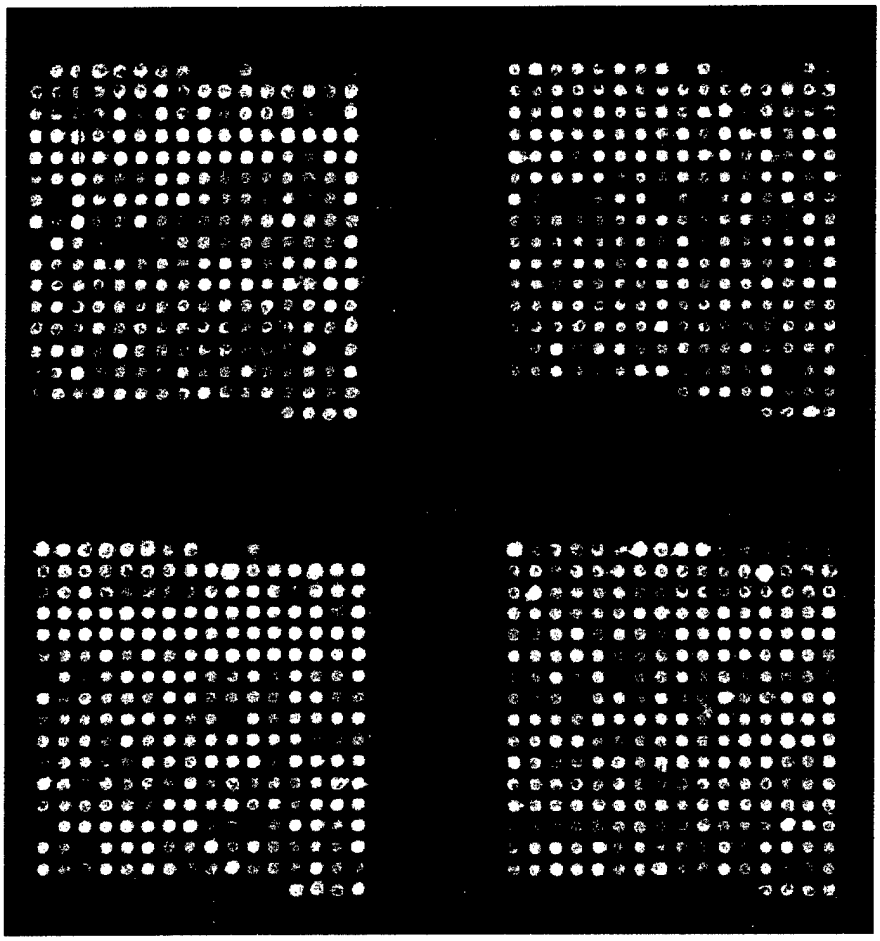
Cell line designation is given to the left. Each dot represents the extent of methylation detected at a given site in ten sequences cloned from bisulfite treated DNA. Black spots have $\geq$60% methylation at the site, white $\leq$50% with the actual number indicated. Blank spots are unmethylated in all samples tested. The region between –1435 and –1099 contains a series of Alu repeats.
Expression data is presented in Table I.

Task 4. Complete collection of CpG island and expression microarray data for MCF10A, MCF10AT and MCF10DCIS.C, cells. Sequencing and identification mRNAs and CpG islands whose expression or methylation differs between the cell lines. (Months 8-18).

This task has been initiated, but has been slowed by the need for development of better probes (above). We anticipate completion of this aim within the next four-six months as all DNAs and RNAs have been prepared and arrays are ready to be probed and analyzed.

A CpG island microarray showing the comparison between methylation of MCF10AT and MCF10DCIS.com is shown below. Analysis is underway. In this experiment MCF10AT DNA was cut with MseI, linkers were added, the DNA cut with BstUI and amplified by PCR[14]. The PCR product was cleaved with a second enzyme and a new linker containing a T7 promoter was added. RNA probe was synthesized using a nucleotide mix with aminoallyl UTP and conjugated with Cy3. The same process was carried out using MCF10DCIS.com DNA but conjugation was with Cy5. Yellow spots indicate that both cells lines have the same extent of methylation; green spots that MCF10DCIS has lost methylation relative to MCF10AT

and red spots that MCF10DCIS has gained methylation at sites that are unmethylated in MCF10AT.



Task 5. Complete analysis of methylation and expression of candidate genes in laser captured cells from lesions at different stages of progression. (Months 16-28).

As described in section 2, methylation analysis of two genes has already been completed. Analysis of the remaining five genes in DCIS and of all 7 genes in areas of tumors with different grades of progression will be completed in the next 4-6 months.

Task 6. Complete collection and analysis of CpG island and expression microarray data for laser captured cells from lesions at different stages of progression and compare with results from MCF10A, MCF10AT and MCF10DCIS.com cells. (Months 24-32).

Once we have verified that changes in methylation detected by our RNA probes are consistent with our bisulfite sequencing data over the next 2-3 months, this aspect of the work should be possible to complete within 6 mos. Data interpretation and identification of novel methylated genes will be carried out in Months 30-36.

KEY RESEARCH ACCOMPLISHMENTS

- Completed and expanded analysis of the methylation patterns of seven known CpG islands in 6 cell lines representative of different stages in the MCF10A model of breast cancer progression.
- Quantitatively determined levels of mRNA for all seven genes
- Compared results for detection of DNA methylation obtained by methylation specific PCR and CpG island methylation with bisulfite sequencing.
- Analyzed of methylation and expression of key tumor suppressor genes in microdissected tumor tissue from highly tumorigenic MCF10AT derivative lines by methylation specific Q-PCR.
- Developed new method of preparing probes for CpG island microarrays.

III. REPORTABLE OUTCOMES

None. A ms. reported as in preparation last year was held back to allow further analysis of NOEY2 and TIMP3 methylation by bisulfite sequencing (above) and to complete methylation specific PCR. It will be submitted in the next 12 mos.

IV. CONCLUSIONS

The major conclusions from the work completed on this project to date are:
1.) That although there are some differences CpG island methylation in the seven genes examined in MCF10AT family cell lines, none can be linked to differences in mRNA levels between the lines representative of different stages of progression. This is best exemplified by the results with TIMP-3.
2) That there are significant differences in methylation of the CpG island of GSTP1 that develop in all of the DCIS areas of tumors formed by these lines, i.e., methylation is detected where there is no methylation in the corresponding cell line. Evaluation of the other candidate genes is underway, as is the evaluation of mRNA expression.
3) Proof of the starting hypothesis will require examination of global CpG island methylation by microarray as proposed.

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